

REMARKS

The Office Action dated December 15, 2008, has been received and carefully noted. The above amendments and the following remarks are being submitted as a full and complete response thereto.

Claims 1-26 are rejected. Claims 1, 6-7, 11-12, 15-18, and 20-21 have been amended herein. Claims 2-4 and 22-26 have been cancelled without prejudice to or disclaimer of the subject matter contained therein. Thus, claims 1 and 5-21 are pending in this application. Support for the amendments may be found in the specification as originally filed. Applicants submit that no new matter is added. Applicants respectfully request reconsideration and withdrawal of the outstanding rejections.

Specification

The specification was objected to for containing typographical errors. A substitute specification is being submitted herewith in order to correct these typographical errors. Accordingly, Applicants respectfully request withdrawal of the objection to the specification.

Claim Objections

Claims 2-4 and 7 were objected to for typographical errors. Claims 2-4 have been cancelled, and 7 has been amended. Accordingly, Applicants respectfully request withdrawal of the objection to claims 2-4 and 7.

Rejections under 35 U.S.C. §101

Claims 22-26 were rejected under 35 U.S.C. § 101 because they were allegedly not presented using the preferred format for a process claim. Without conceding the propriety of this rejection, Applicants submit that it is moot in view of the cancellation of claims 22-26.

Rejections under 35 U.S.C. §112, second paragraph

Claims 3, 4, 17, 18, and 21-26 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

Claims 3 and 4 (which have been cancelled, and their subject matter has been incorporated into claim 1) were rejected as allegedly having unclear scope with regard to the alleged cross-reactivity between the two isoforms of SEQ ID NO: 2 recited in the claims. The Office Action indicated that the rejection could be overcome by indicating which asparagine (N) residue is glycosylated. Applicants submit that there is only one asparagine residue in SEQ ID NO: 2, and that claim 4 indicated that the isoform was glycosylated, whereas claim 3 indicated that it was not glycosylated. The Office Action admits that the specification (on p. 10, lines 23-26) indicates the residue of SEQ ID NO: 2 that is glycosylated. Accordingly, Applicants submit that this rejection appears misplaced, because one of ordinary skill in the art would understand the difference between the glycosylated version of SEQ ID NO: 2 and the non-glycosylated version of SEQ ID NO: 2 upon consultation of the specification.

However, without conceding the propriety of this rejection, Applicants submit that

claim 1 now indicates that the antigenic epitope of the non glycosylated nuclear isoform is selected from the group of amino acid sequences consisting of QFNWVSRLANLTQGEDQK (SEQ ID No 1), and non glycosylated TKLKELPGVCNETMMALWEE (SEQ ID No 2), and the antigenic epitope of the glycosylated cytoplasmic isoform is selected from the group of amino acid sequences consisting of TKLKELPGVCNETMMALWEE (SEQ ID No 2) glycosylated at its N residue; TNEERKTLLSNLEELAK (SEQ ID No 3), and METVAEKALQEYRKK (SEQ ID No 4).

Claims 18 and 21 were rejected for lack of antecedent basis for "tumours" in claims 18 and 21 and "diagnostic kit" in claim 21. Applicants submit that the dependencies of these claims has been corrected, such that claims 18 and 21 now depend from claims 16 and 20, respectively.

Claims 22-26 were rejected for being vague and indefinite for allegedly failing to set forth active, positive steps that define the claimed method. Without conceding the propriety of this rejection, Applicants submit that it is moot in view of the cancellation of claims 22-26.

Claims 17 and 26 were rejected for being vague and indefinite for reciting "liquor" without indicating where the liquor comes from. In response, Applicants submit that one skilled in the art having the present specification before him would understand that the "liquor" may be derived from any of a number of sources within the body (e.g., amniotic fluid, cerebrospinal fluid, etc.). The term "liquor" is therefore not vague or indefinite.

In view of the amendments and remarks set forth above, Applicants respectfully request withdrawal of the § 112, second paragraph, rejection of claims 3, 4, 17, 18, and 21-26.

Rejections under 35 U.S.C. §112, First Paragraph (Written Description)

Claims 1-26 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

The Office Action takes the position that claims 1, 2, 5-10, and 15-26, given their broadest reasonable interpretation, encompass antibodies that bind to all variations of clusterin, including clusterin in different tissues and species. This statement is at least partially in error, as these claims specifically recite that the clusterin is “human” clusterin, and therefore does not include clusterin in other species.

The Office Action also takes the position that the description of antibodies that bind SEQ ID NOS: 1-4 fails to adequately describe the genus of antibodies claimed. Without conceding the propriety of this rejection, Applicants submit that it is moot because claim 1 has been amended to recite that the oligoclonal antibodies are able to recognize and bind the antigenic epitope of at least one glycosylated cytoplasmic or non glycosylated nuclear isoform of human clusterin, where the antigenic epitopes are selected from SEQ ID NOS: 1-4.

The Office Action further alleged that claims 1-26 lack sufficient written description because the amount of sequence variability allowed for by the claim to “derivatives thereof” is too large. Without conceding the propriety of this rejection, Applicants submit that it is moot because the term “derivatives thereof” has been

cancelled from the claims.

In view of the amendments and remarks set forth above, Applicants respectfully request withdrawal of the written description rejection of claims 1-26 under §112, first paragraph.

Rejections under 35 U.S.C. §112, First Paragraph (Enablement)

Claims 16 and 23 are rejected under 35 U.S.C. §112, first paragraph, for allegedly lacking enablement for analysis of malignancy in cancers other than colon cancer.

The Office Action took the position that claims 16 and 23 lack enablement for the analysis of malignancy in cancers other than colon cancer, because undue experimentation would allegedly be required for one of ordinary skill in the art to be able to practice the claimed method for cancers other than colon cancer.

Applicants submit that claim 23 has been cancelled. With respect to claim 16, Applicants submit that the immunological method of the invention is suitable for the diagnosis and the prediction of the malignancy of any tumor characterized by expression of clusterin, not only for colon cancer.

Applicants are providing herewith scientific articles concerning the relationship between the malignancy of different kinds of tumors and clusterin expression. These articles demonstrate that clusterin is expressed by a number of different types of tumors, including, but not limited to, tumors of the bladder, breast, lung, kidney, colon/rectum, and prostate:

- Sun et al., "Response of Bladder Carcinoma Cells to TRAIL and Antisense Oligonucleotide, Bcl-2 or Clusterin Treatments." *J Urol*. 2009 Jan 19. (E-published ahead of print). (Attached as Exhibit A.)
- Chia et al., "Phase II trial of OGX-011 in combination with docetaxel in metastatic breast cancer." *Clin Cancer Res*. 2009 Jan 15; 15(2):708-13. (Attached as Exhibit B.)
- Chi et al., "Custirsen (OGX-011): a second-generation antisense inhibitor of clusterin for the treatment of cancer." *Expert Opin Investig Drugs*. 2008 Dec; 17(12):1955-62. (Attached as Exhibit C.)
- Muramaki et al., "Chemosensitization of gemcitabine-resistant human bladder cancer cell line both in vitro and in vivo using antisense oligonucleotide targeting the anti-apoptotic gene, clusterin." *BJU Int*. 2009 Feb; 103(3):384-90. (Attached as Exhibit D.)
- Olsen et al., "Clusterin expression in cutaneous CD30-positive lymphoproliferative disorders and their histologic simulants." *J. Cutan. Pathol*. 2009; 36:302-307. (Attached as Exhibit E.)
- Trougakos et al., "Advances and Challenges in Basic and Translational Research on Clusterin." *Cancer Res*. 2009; 69(2):403-406. (Attached as Exhibit F.)
- Pucci et al., "Interleukin-6 affects cell death escaping mechanisms acting on Bax-Ku70-Clusterin interactions in human colon cancer progression." *Cell Cycle* 2009; 8(3):473-481. (Attached as Exhibit G.)

For completeness sake, Applicants would like to highlight that the methods of the presently-claimed invention permit discrimination between healthy individuals and cancer patients using a non-invasive test that is carried out on a biological sample, such as blood.

Another important aspect of the present invention is that, in order to discriminate between healthy individuals and cancer patients, the biological sample to be tested is not taken by invasive methods, such as a biopsy. For example, in the case of an individual suspected of having colon cancer, it is possible to avoid a colon endoscopy. The presently-claimed antibodies directed to the specific epitopes belonging to the cytoplasmic clusterin isoform (secreted isoform) permit non-invasive pre-screening of

biological samples, such as blood or stool sample. This allows direct discrimination between healthy individuals and cancer patients by a simple, non-invasive pre-screening test, thereby allowing healthy individuals to avoid undergoing invasive procedures.

Applicants have discovered as part of the present invention that SEQ ID NOS: 1-4 show a surprising ability to recognize and bind to the antigenic epitopes of the isoforms of human clusterin in a selective and specific way, so that it is possible to discriminate healthy individuals from patients having a variety of different cancers that are characterized by clusterin expression.

Accordingly, in view of the disclosure provided in Applicants' specification and the general knowledge of those skilled in the art at the time the presently-claimed invention was made, a person skilled in the art would be able to carry out the method of claim 16 in order to diagnose tumors characterized by clusterin expression, and predict their malignancy grade, without undue experimentation.

In view of the amendments and remarks set forth above, Applicants respectfully request withdrawal of the enablement rejection of claims 16 and 23 under §112, first paragraph.

Rejections under 35 U.S.C. §102

Claims 1, 2, 5-10, 15, 19, 20-22, and 24 were rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Lakins et al. (*Journal of Biological Chemistry*, 1998, Vol. 273, pages 27887-27895, hereinafter "Lakins"). Applicants respectfully traverse this rejection.

The Office Action alleged that Lakins discloses two polyclonal antibodies made from peptides consisting of 10-20 amino acids of human clusterin that bind non-glycosylated and glycosylated protein respectively. The Office Action also alleged that Lakins discloses detection of the polyclonal antibodies using a reaction with a secondary antibody.

The positions taken in the Office Action are at least partially mistaken, as Lakins does not disclose human clusterin or antibodies specific to human clusterin, but instead only discloses rat clusterin and antibodies specific to rat clusterin.

Lakins produced polyclonal antibodies (named 301) directed against multiple epitopes of rat clusterin. In particular, the sequence NGDRIDSLLESDRQQSQ used by Lakins (residues 133-148 of rat Clu NM_053021) differs from the homo sapiens clusterin sequence: NGDRIDSLLENDRQQTH (residues 206-223 of homo sapiens Clu, transcript variant 1). They are not identical, and differ at the two underlined amino acids. Thus, the peptide mixture used by Lakins (in which 4 epitopes were selected – not one – and the epitopes did not match human clusterin) gives rise to an antibody pool that is not specific for the tertiary structure of human clusterin (glycosylated and not glycosylated) isoforms.

Accordingly, Lakins fails to anticipate the claims. Further, Applicants submit that Lakins does not disclose or suggest that the antigenic epitopes of human clusterin are selected from SEQ ID NOS: 1-4, as is now recited in independent claim 1.

Accordingly, Applicants respectfully submit that Lakins does not anticipate independent claim 1. As claims 5-10, 15, 19, 20-22, and 24 depend from allowable claim 1, the Applicants submit that these claims are likewise allowable.

For at least the above reasons, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 2, 5-10, 15, 19, 20-22, and 24 under 35 U.S.C. §102(b) over Lakins.

Rejections under 35 U.S.C. §103

Claims 7-9 are rejected under 35 U.S.C. §103(a) as being unpatentable over Lakins in view of Kerr and Thorpe (*Immunochemistry LabFax*, 1994, pages 118, 128-129, 158, hereinafter "Kerr"). Applicants respectfully traverse this rejection.

The deficiencies of Lakins with respect to the presently-claimed invention are discussed above.

The Office Action alleged that while Lakins does teach tagging anti-clusterin polyclonal antibodies with biotin, it does not specifically teach using other tags. Kerr is said to teach antibody tagging with radiolabels, fluorescein, horseradish peroxidase, and alkaline phosphatase, and their use in antigen-antibody visualization. Based on this disclosure, the Office Action concluded that it would be obvious to combine the teachings of Lakins and Kerr, and that one would have been motivated to do so.

Applicants submit that the Kerr reference fails to remedy the deficiencies of Lakins with respect to the presently-claimed invention, as discussed above.

In addition, Applicants are providing herewith the Declaration under 37 C.F.R. § 1.132 of Luigi G. Spagnoli (attached as Exhibit H). The data presented in the Declaration demonstrates the advantages of the oligoclonal antibodies to SEQ ID NOS: 2-4 in comparison to anti-rat clusterin antibodies to the peptide NGDRIDSLLESDRQQSQ, which was one of four peptides derived from the alpha chain

of the mature rat clusterin protein that was used to generate antibodies in Lakins (designated as "SEQ ID NO: 5" in the Declaration).

As described in the Declaration, Figure 1 shows the strikingly lower level of sensitivity and specificity of the antibody raised against SEQ ID NO: 5, as compared to the antibodies raised against SEQ ID NOS: 2-4. See Declaration, paragraphs 10-11. This comparison was carried out using SEQ ID NO: 5, rather than the other three peptides disclosed in Lakins, because SEQ ID NO: 5 shows higher homology to the human clusterin counterpart.

As shown in Figure 1 of the Declaration, after the 4th immunization booster, ELISA assays were conducted to demonstrate that the antibody obtained using SEQ ID NO: 5 (raised against the glycosylated form of rat clusterin) cannot be used to discriminate between healthy individuals and cancer patients when used to test human serum. These results confirm the disclosure found in Lakins, in which Western blot analysis was performed on serum samples, but the antibodies disclosed in Lakins were unable to detect serum clusterin. See page 27891, second column, "Characterization of Serum Clusterin."

Serum clusterin is highly glycosylated, and in the denaturing conditions of a Western blot analysis, the glycosylation-dependent tertiary conformation is lost. The presently-claimed antibodies against the cytoplasmic clusterin (SEQ ID NO: 4) recognize the native form. In fact, the antibodies produced by Lakins also cannot detect clusterin when using immunohistochemistry methods.

Conversely, the epitopes chosen by the present inventors provide strong sensitivity for detecting clusterin isoforms, and evaluating clusterin increases in early stage cancer patients with higher sensitivity.

In view of this experimental data, it would not have been obvious for one skilled in the art to modify the teachings of Lakins and Kerr to arrive at the presently claimed invention.

For at least the above reasons, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 7-9 under 35 U.S.C. § 103(a) over Lakins in view of Kerr.

CONCLUSION

Applicants respectfully submit that this application is in condition for allowance and such action is earnestly solicited. If the Examiner believes that anything further is desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact Applicants' undersigned representative at the telephone number listed below to schedule a personal or telephone interview to discuss any remaining issues.

In the event that this paper is not being timely filed, the Applicants respectfully petition for an appropriate extension of time. Any fees for such an extension, together with any additional fees that may be due with respect to this paper, may be charged to Counsel's Deposit Account Number 01-2300, referencing Docket Number 026073-00007.

Respectfully submitted,



Dawn C. Russell
Attorney for Applicants
Registration Number 44,751

Customer Number 004372
ARENT FOX LLP
1050 Connecticut Avenue, NW
Suite 400
Washington, DC 20036-5339
Telephone: 202-857-6000
Fax: 202-638-4810

DCR:vmh

Enclosures: Marked-Up Substitute Specification (20 pages)
Clean Copy Substitute Specification (20 pages)
Exhibits A-G (Articles)
Exhibit H (Declaration under 37 C.F.R. § 1.132 of Luigi G. Spagnoli)

MARKED-UP
SUBSTITUTE SPECIFICATION

5 ANTI-CLUSTERIN OLIGOCLONAL ANTIBODIES FOR DIAGNOSIS AND
PREDICTION OF THE AGGRESSIVENESS OF TUMOURS,
DIAGNOSTIC METHOD AND RELATED KITS

10 This invention concerns anti-clusterin oligoclonal antibodies for
tumour diagnosis and prediction of the malignancy grade of the tumour,
diagnostic method and related kits.

In particular, the invention concerns anti-clusterin oligoclonal antibodies
selective and specific for diagnosis of the onset or the relapse and the
prediction of aggressiveness of tumours such as colon, breast, prostate,
testis and ovary carcinomas, tumours of the central nervous system (SNC)
15 and of the haemo-lymphopoietic system.

At present, colon cancer is second only to lung cancer in men
and to breast carcinoma in women, for incidence and mortality in western
countries.

20 In Italy, the incidence of colon cancer is: 40 new cases every
100,000 inhabitants (30,000 new cases per year). The mortality is 18,000
cases per year.

The higher incidence per age is observed between the sixth
and seventieth decade, while 60% of the patients survive up to five years.
The most important reason for the low percentage of recoveries is due to
25 the fact that when the primary tumour is removed, a high number of
patients have already developed micro-metastases, principally at liver.
Therefore, methods for early screening are requested. At present, the
early diagnosis protocols (secondary prevention) consist of rectal
exploration, determination of fecal occult blood, recto-sigmoidoscopy or
30 pan-colonoscopy, periodically performed on individuals of 45 years of age
and older and non-symptomatic. Periodic pan-colonoscopy is the only
procedure for early diagnosis of neoplasia, on individuals that are part of
the so called "at risk population", that is individuals with positive familiar

anamnesis for colorectal cancer, patients with already a neoplasia or affected by syndrome with an high risk of neoplasia insurgence.

Obtaining more information on the molecular alterations of a tumour would help to subdivide patients affected by the same tumour in groups with different prognosis, and then perform the specific therapeutic protocols. Moreover, this molecular alteration could be the target of a specific therapy that would revolutionize the pharmacological therapy of cancer.

Recent studies have demonstrated an increase in clusterin expression in breast carcinoma (Redondo et al, 2000), suggesting a possible role of this protein in tumour progression.

Clusterin is an eterodimeric (α and β chains) ubiquitous glycoprotein implicated in a large number of physiological processes and in the control of cellular proliferation (Murphy et al, 1988; Aronow et al, 1993; Fratelli et al, 1996; Ho et al, 1998; Humphreys et al, 1999; O'Sullivan et al, 2003; Zhou et al, 2002; Bettuzzi et al, 2002). A number of isoforms have been characterized that differ in the grade of glycosilation and in their function.

The authors of the present invention have studied clusterin expression (Pucci et al, 2004) in order to elucidate its role in tumour progression, in particular in the adenoma-carcinoma sequence of colorectal cancer, a well known human tumour model. In this context, the observation of 35 samples of human intestinal mucosa, such as endoscopic biopsies and surgical specimens has shown a different modulation of clusterin isoforms in the various cell compartments, directly related to tumour progression. In fact, in healthy colon mucosa it has been observed clusterin with an exclusive nuclear localization, that regulates cell cycle progression and apoptosis induction. On the other hand, in carcinogenesis and in tumour progression a clear increase in the cytoplasmic isoform expression has been observed, while the nuclear isoform is lacking.

Evidences about the protein localization have helped recognize two principal isoforms: a nuclear ~~non-glycosilated~~ non-glycosylated isoform in healthy mucosa and in adenomas; a cytoplasmic ~~glyeosilated~~ glycosylated isoform highly expressed in neoplastic cells and absent in healthy mucosa.

The results concerning the modulation of different clusterin isoforms in the tumourigenesis of colorectal cancer shed light on the controversial data on increase of clusterin in tumours and on the role of the protein in apoptosis induction, suggesting a possible role of clusterin as a potential new marker for the diagnosis and prognosis of colorectal cancer.

The use of clusterin as a diagnostic marker in some pathological conditions such as diabetes mellitus of type II and some coronary pathologies has already been described in the Greek patent GR 20020100196. The Greek document describes an ELISA method which uses two commercial antibodies (one of which is conjugated with the HRP enzyme), for the quantitative determination of serum ApoJ/Clusterin levels, related to the above mentioned pathologies.

There was just one previous attempt to determine ApoJ by ELISA in tumoural pathologies, specifically in the blood of prostate carcinoma patients (Morrisey et al, 2001), as referred in the Greek document.

On the basis of the above, it is clear the need of new materials and methods for diagnosis and prediction of the grading and clinical staging of some tumours i.e. colorectal cancer, that would overcome the restrictions of the invasive methods used at present.

The authors of this invention have highlighted that the appearance and the progressive increase of the clusterin cytoplasmic isoform in tumours correlates to a significant increase of clusterin in the serum of patients affected by tumoural pathologies and in particular by colorectal cancer.

On the basis of this observation, the authors have tested a new method of immuno-dosage of clusterin with the use of antibodies that can recognize specifically and selectively the cytoplasmic and nuclear isoforms of clusterin, for diagnosis of the molecular alterations that could define a prognosis and give therapeutic indications.

In fact, as already described the disappearance of clusterin nuclear ~~non-glycosilated~~ non-glycosylated isoform and the preferential expression of a second isoform completely glycosilated glycosylated and

cytoplasmic seems to be directly related to the aggressiveness of the tumour and to its metastatic potential (Pucci et al, 2004).

In particular, this dosage method of the two clusterin isoforms, using antibodies according to the present invention, not only represents a non invasive tool, but it is also an easy and not expensive approach for the patient's follow-up and it could help formulate a "biological aggressiveness index" of the neoplasia, that would help standardizing this method. In particular, the authors have tested a standard curve, where the protein of reference is not the purified clusterin but a peptide synthesized on the basis of the new epitope chosen for immunization.

Serological techniques such as ELISA and/or immunofluorescence used presently need monoclonal antibodies, because of the difficulty in obtaining specific sera.

The authors of this invention have now obtained sera containing oligoclonal antibodies specific and selective for the selected epitopes, belonging to clusterin cytoplasmic and nuclear isoforms. These oligoclonal antibodies have not the disadvantages of reproducibility, production costs and conservation of monoclonal antibodies obtained from hybridomas. Indeed, selection and conservation of an hybridoma has a number of limitations: low reproducibility of the clone and antibody specificity toward the epitope, technical difficulty in the production of the hybridoma, high cost for the maintenance and difficult conservation of samples in time.

Moreover, the manufacturing of oligoclonal antibodies based on the selection of very short antigenic epitopes (synthesized on solid phase) permits the determination of a small repertoire of antibodies that are comparable to monoclonal antibodies, considering the characteristics of specificity and affinity. According to this invention and for an easier application of the oligoclonal antibodies, the antigenic sequences of the epitopes have been chosen among the most highly conserved both in human and mouse, permitting a wider application for animal model research. Moreover, this choice allows to minimize the binding with human proteins different from clusterin. The selection of these epitopes has also been made to guarantee the maximum specificity and efficiency and the

minimum cross-reactivity of the oligoclonal antibody with the various ~~glycosilated~~ glycosylated isoforms of cytoplasmic clusterin and to obtain highly specific and selective antibodies for the ~~non-glycosilated~~ non-glycosylated nuclear isoform.

5 It must be pointed out that the commercial anti-clusterin antibodies are not able to identify this nuclear isoforms, because the immunization used for their preparation is done administering the proteins purified from serum, that the authors have recently demonstrated it contains exclusively the ~~glycosilated~~ glycosylated isoform of the protein.

10 The authors of the present invention have previously found that in the colorectal cancer the secreted isoform of cytoplasmic clusterin is released in the extra-cellular space and in the lumen of colon, thus an increased level of clusterin also in the stools of the colon carcinoma patients could be expected. Therefore, the dosage of clusterin can be carried out, further
15 than that in peripheral blood, also in the stools of the colorectal cancer patients with a blood-stool cross-shaped test, highly specific for the colon carcinoma. In this manner, the problem of the interference of the increased level of clusterin due to other not tumoural or tumoural diseases (cancer of breast, prostate, testicle, ovary, SNC, haemo-lymphopoietic system) is abolished.

20 Therefore, it is an object of the present invention oligoclonal antibodies which are able to recognize and to bind in a specific and selective manner the antigenic epitope of at least one isoform of the clusterin, said antigenic epitope being characterised by a length comprised between 10 and 20
25 amminoacidic residues. The clusterin isoform which is recognized by the anti-clusterin oligoclonal antibodies can be the ~~not-glycosilated~~ not-glycosylated nuclear or the ~~glycosilated~~ glycosylated cytoplasmic one. According to the present invention, these oligoclonal antibodies discriminate between different clusterin isoforms. In particular, the
30 antigenic epitope selected to produce the oligoclonal antibodies against the nuclear ~~not-glycosilated~~ not-glycosylated clusterin isoform comprises an amminoacidic sequence selected from the group consisting of:

QFNWVSRLANTQGEDQK (SEQ ID No.1);

TKLKELPGVCNETMMALWEE (SEQ ID No. 2);

and derivatives thereof obtained by deletion, substitution or addition of one or more aminoacids, which maintain the same immunogenic property of the original epitope. The antigenic epitope
 5 chosen to produce the oligoclonal antibodies directed against the cytoplasmic ~~glycosilated~~ glycosylated isoform can comprise one of the following aminoacidic sequences:

TKLKELPGVCNETMMALWEE(SEQ ID No. 2);

TNEERKTLLSNLEEK (SEQ ID No. 3);

10 METVAEKALQEYRKK (SEQ ID No. 4);

and their derivatives obtained by the means of deletion, substitution either addition of one or more aminoacids that maintain the same immunogenic property of the original epitope.

According to the present invention, the oligoclonal antibodies
 15 can be tagged preferably with a fluoro-chrome, a radioactive isotope, an enzyme, biotin or a chemiluminescent substance. The fluoro-chrome being the fluorescein or phycoeritrin, or rodamine, or the texas red, or cumarin; the enzyme being the peroxidase of radish (HRP) or alkaline phosphatase; the radioactive isotope could be ^{14}C , or ^3H ; the chemi-luminescent
 20 substance can be luciferin.

They constitute further subjects of the present invention antigenic epitopes belonging to at least one of the clusterin isoforms, cytoplasmic and/or nuclear, comprising at least one of the following aminoacidic sequences:

25 QFNWVSRLANLTQGEDQK (SEQ ID No. 1);

TKLKELPGVCNETMMALWEE (SEQ ID No. 2);

TNEERKTLLSNLEEK (SEQ ID No. 3);

METVAEKALQEYRKK (SEQ ID No. 4);

and their derivatives obtained by deletion, substitution or
 30 addition of one or more aminoacidic residues that maintain immunogenic properties of the original epitope.

Further issue of the present invention is represented by the method of preparation of the oligoclonal antibodies, how previously described, comprising the following phases:

- 5 a) Solid phase synthesis of at least one of the antigenic epitopes, described from above;
- b) Conjugation of at least one antigenic epitope with a protein carrier as immunogen, preferably bovine serum albumin;
- c) Immunization in animal with the immunogen epitope (carrier-conjugated), in complete Freund adjuvant;
- 10 d) Withdrawal of the serum from animal, preferably rabbit, and purification of the oligoclonal antibodies, e.g. by affinity chromatography.

Further issue of the present invention is an immunological assay performed to detect clusterin protein levels in biological samples for diagnosis of tumour and prediction of its malignancy grade. The immunological method comprises the following phases:

- 15 a) Extraction of the proteins from the biological sample, such as blood, stool, seminal fluid, pleural and ascitic effusions, urine, liquor;
- b) Incubation in timely condition of the protein extracted with at least one of the oligoclonal antibodies above described, to form an antigen-antibody complex;
- 20 c) Qualitative and quantitative detection of this antigen-antibody complex.

In particular, the immunological method according to this invention allows the diagnosis of tumours such as colorectal, breast, prostate, testis and ovary carcinomas, tumours of the Central Nervous System and of the haemo-lymphopoietic system.

The determination in phase c) can be performed by using one of the following techniques: ELISA, Western Blot, RIA, immunohistochemistry detected with fluorochromes (immunofluorescence) or enzymatic method, or with a combination of these techniques.

This invention also includes a diagnostic kit for the diagnosis of neoplasia and for the prediction of its malignancy grade, comprising at

least one of the oligoclonal antibodies above described. The tumors that can be detected are, for example, colorectal, breast, prostate, testis and ovary carcinomas, tumours of the Central Nervous System and of the haemo-lymphopoietic system. Finally, a further topic of the present invention is represented by the use of at least one of the oligoclonal antibodies described previously, for the qualitative and quantitative determination of the levels of at least one clusterin isoforms, in a biological sample such as blood, stool, seminal liquid, pleural and ascitic effusion, urine, liquor, for diagnosis of tumours (e.g. colorectal, breast, prostate, testis, ovary carcinomas, tumours of the Central Nervous System and of the haemo-lymphopoietic system) and prediction of their aggressiveness.

The qualitative or quantitative determination can be carried out by using one of the following techniques: ELISA, Western Blot, RIA, immunohistochemistry detected with fluorochromes (immunofluorescence) or enzymatic method, or with a combination of them.

To better clarify, the term "oligoclonal antibodies" means a limited repertoire of polyclonal antibodies obtained by later on cycles of specific immunization, at the conclusion of which antibodies will be obtained that are comparable to monoclonal antibodies, considering the characteristics of specificity and affinity.

The present invention will be now described to illustrate it, but not to limit it, according to the preferred embodiments, with special reference to the attached figures, in which:

Fig.1 shows the results obtained by Western blot analysis of the nuclear form of clusterin; in panel A, antibody against a not ~~glycosilated~~ glycosylated antigenic epitope of the clusterin α -chain, SEQ ID No 1 has been used; in panel B the antibody has been obtained by immunization with a not ~~glycosilated~~ glycosylated antigenic epitope of the clusterin β -chain, SEQ ID No 2;

Fig.2 shows the Western blot analysis for ~~glycosilated~~ glycosylated cytoplasmic clusterin; in panel A the antibody was directed against a ~~glycosilated~~ glycosylated epitope of clusterin β -chain, SEQ ID No2; in panel B the antibody was generated by immunization against an

antigenic epitope of β -chain, SEQ ID No 3; in panel C the antibody was directed against an antigenic epitope of clusterin α -chain, SEQ ID No 4;

Fig 3 shows the results obtained by ELISA, performed to analyze nuclear and secreted clusterin, in tumoural tissues and in corresponding normal samples; panel A shows the results obtained using the antibody directed against not glycosilated glycosylated nuclear clusterin isoform (antigenic epitope SEQ ID No1); panel B shows the detection with antibody against the secreted glycosilated glycosylated clusterin (antigenic epitope SEQ ID No4).

Fig 4 shows the dosage of clusterin ($\mu\text{g/ml}$) by ELISA in the culture supernatant fraction of *ex vivo*-isolated tumoural cells, compared to cells from healthy colonic mucosa (number of cells seeded: 10×10^7).

EXAMPLE 1: Immunization procedure and anti-clusterin oligoclonal antibodies production.

In order to obtain a specific anti-clusterin oligoclonal antibody the standard immunization technique has been used. The method consists on to immunize rabbit or other animal against a specific immunogenic epitope of the protein. In the serum of the immunized animal will be found polyclonal antibodies produced from different clones of activated plasmacells.

MATERIALS AND METHODS:

Immunogen production: synthesis and purification

Short Clusterin-specific antigenic sequences synthesized in solid phase and having a length from 10 to 20 amminoacids have been used.

A comparison between human and murine (*mus musculus*) amminoacidic sequence has been performed and the antigenic epitopes have been characterized using the algorithm of Kolaskar A.S. and Tongaonkar P.C. (1990), accessible from the program "Antigenic Peptide Prediction" (www.mifoundation.org).

The sequences, shared between man and mouse (the antibodies so can be employed in the mouse and in the rat) were chosen to contain an antigenic epitope that could be potentially glycosilated

glycosylated. Two likely sites of protein glycosylation allowing the discrimination between the two different isoforms (glycosylated glycosylated or not glycosylated glycosylated) were selected and the shorter sequence was chosen in order to obtain the greatest specificity and efficiency and the smaller cross-reactivity with the different glycosylated glycosylated isoforms of clusterin. In fact, the immunization performed with not glycosylated small epitopes agreed to obtain specific and selective antibodies for the recognition of the nuclear form of clusterin (not glycosylated).

10 The identification of the different isoforms of clusterin was obtained in Western Blot analysis by denaturing the proteins, in a protein extract from tissue. The selected aminoacidic sequences are: QFNWVSRLANLTQGEDQK (SEQ ID No 1), that consists in a not-glycosylated not-glycosylated antigenic peptide of the α chain that recognize the nuclear form of clusterin; TKLKELPGVCNETMMALWEE (SEQ ID No 2), that consists in an not-glycosylated not-glycosylated antigenic peptide of the β chain (used to produce the second antibody against the nuclear isoform), including the glycosylation site in the position 103 (underscored).

20 In order to obtain the immunogenicity, these peptides were linked to a carrier protein such as the Bovine serum albumin, (BSA), highly stable and soluble in the plasma. A commercial kit "Imject® Immunogen EDC Conjugation kit", Pierce (Rockford, IL, US) was used.

25 The animal model was selected taking into account the high inter-specie homology and conservation of the protein sequence. Three epitopes were used for the rabbit immunization because of the poor immunogenicity of the antigen, despite the conjugation with BSA and administration of complete Freund adjuvant.

In particular, the following three epitopes have been used:

30 TKLKELPGVCNETMMALWEE (SEQ ID No 2) that consists in a glycosylated glycosylated antigenic peptide of the β chain used to produce the second antibody against the cytoplasmic iso-form including the glycosylation site in the position 103 (underscored);

TNEERKTLLSNLEEK (SEQ ID No 3) that consists in a peptidic sequence of the β chain used to identify the cytoplasmic form; METVAEKALQEYRKK (SEQ ID No 4) that is the antigenic peptide of the α chain, used to determine the presence of the cytoplasmic form.

5 The immunogenic solution consisting in carrier-linked antigenic peptide and complete Freund adjuvant was prepared in a volume of 150 μ l and inoculated under-skin in rabbit. For every not-glycosylated not-glycosylated or glycosylated glycosylated peptide synthesized in solid phase, three rabbits were immunized following the
10 protocol of the Corning Hazleton Virginia (Wien, VA). In particular two of the peptides selected have been used solely or in combination, in a 1:1 ratio.

 Moreover, an epitope of the clusterin β chain was used that did not include potentially glycosylated glycosylated sites, in order to obtain an
15 antibody able to detect all the clusterin isoforms.

 The immunization with the epitopes containing potentially glycosylated glycosylated sites allowed to discriminate both by Western blot and ELISA the cytoplasmic isoform of clusterin, a new marker of
20 tumour aggressiveness and metastatic potential. After 7 days from the first immunization with the immunogenic peptides the cyclic immunization were performed ones every 7 days, for 6 times, in order to obtain an antibody repertoire with high specificity and affinity. After 3 cycles of immunization the antibody production has been analysed by ELISA, a technique used to
25 monitor the presence of a specific antibody in the serum.

 The serum was obtained from 5 ml of blood of the immunized rabbit. The control serum consisted in the pre-immune serum of the same animal withdrawn before the first immunization. The blood for the affinity and specificity test was collected after the 4th, 5th and 6th immunization.

 After the 5th and the 6th immunization the antibodies from the
30 rabbit sera were precipitated with a saturated sulphate ammonia solution and purified by affinity chromatography, using protein A bound to agarose beads (SIGMA). The rabbit IgG were obtained after a wash in acid buffer 0.05M Na₂HPO₄, 0.025M citric acid pH3.

Screening of the anti-serum antibodies and specificity tests

The immune response has been evaluated by ELISA, to the beginning of the third cycle of immunisation.

5 Blood pulled out from three different rabbits was analysed for the evaluation of the adequacy for the ELISA test. In particular, the title of the anti-serum with different dilutions in the range of highest binding level, together with the sensitivity and the specificity of the ELISA have been examined.

10 After the sixth immunisation, the following tests have been performed.

For the binding assays to the different dilution ranges, the distinct antibodies were incubated in complete culture medium (control), or in medium supplemented with serum, in presence of known amounts of the peptide. The percentage of bounded peptide and the corresponding
15 dilution of the antiserum were reported in a diagram and the antiserum dilution binding from 30% to 40% of total peptide has been calculated.

The proper dilution of the antiserum has been used to perform a standard scale as reference extending from 0.03 to 300,000 ng/ml, to evaluate the assay sensitivity.

20 The cross-reactivity with other known lipoproteins: Apo E, Apo AI and the immunogen transport protein (BSA), has been evaluated to the concentrations from 0.50 to 300,000 ng/ml.

The percentage of cross-reactivity has been evaluated using the formula:
50% molar concentration of clusterin peptide/concentration of other protein
25 compound in the assay. The results are shown in Table 1.

Table 1.

	Antibody SEQ ID No 1	Antibody SEQ ID No 2	Antibody SEQ ID No 2	Antibody SEQ ID No 3	Antibody SEQ ID No 4
Clusterin	100	100	100	100	100

ApoAI	<0,001	<0,001	<0,001	<0,004	<0,006
ApoE	<0,001	<0,002	<0,008	<0,002	<0,003
BSA	<0,009	<0,008	<0,01	<0,007	<0,01

Hence, the anti-sera at higher titre and lower cross-reactivity have been chosen.

The oligoclonal antibodies selected after six cycles of immunisation have been used to analyse the expression profiles of clusterin, in protein extracts obtained from murine and human tissue biopsies.

The expression analysis in whole cell lysates with protein extracts from murine and human tissues allowed us to study the protein expression both qualitatively and quantitatively.

By the Western Blotting technique, the oligoclonal antibody (1:1000 dilution) is able to recognize the nuclear clusterin/ApoJ protein, with an almost exclusive affinity for this isoform. In fact, there are revealed a 55 kDa band for the nuclear ~~non-glycosilated~~ non-glycosylated protein (Burkey et al, 1991; Wong et al, 1993; Lakins et al, 1998; Leskov et al, 2003) and a 80 kDa band, which represents the ~~non-glycosilated~~ non-glycosylated precursor protein (holo-protein), in human as well as in mouse. This demonstrates that the antibody does not cross-react with other proteins and therefore is highly specific.

RESULTS

The antibodies against the SEQ ID No.1 and the SEQ ID No.2 epitopes have been tested by denaturing Western Blot for the revelation of the nuclear ~~non-glycosilated~~ non-glycosylated clusterin isoform and to verify their ability to reveal the molecular weights, corresponding to the diverse clusterin isoforms, in a protein lysate.

Indeed, nuclear clusterin involved in cell proliferation regulation and apoptosis induction has been found in healthy colon mucosa. A total protein lysate from healthy colon mucosa has been prepared. The protein amount has been determined by Bradford assay, using a standard curve (BSA) and 15 µg of protein extract have been loaded on a polyacrylamide denaturing gel.

The Western Blot analysis has shown that the two antibodies for the ~~non-glycosylated~~ non-glycosylated form have a high affinity for the nuclear ~~non-glycosylated~~ non-glycosylated form of the protein (50-55 kDa).

In figure 1, the Western Blot for nuclear clusterin is shown. The high affinity antibodies for the ~~non-glycosylated~~ non-glycosylated isoform were obtained in rabbits with a booster after 5 immunizations. "Booster" is the exponential growth phase of immunologic response induced in an animal by the antigen.

The expression levels of cytoplasmic clusterin are very high in aggressive and metastatic colon carcinomas.

Total protein extracts from surgical specimens of colon carcinomas have been prepared. In particular, 15 µg of total proteins have been analyzed by Western Blot. The samples have been loaded in triplicate and analyzed with the three antibodies against the cytoplasmic ~~glycosylated~~ glycosylated isoform (epitopes SEQ ID No 2, 3, 4). Figure 2 shows the Western Blot analysis with each of the three antibodies. As shown, a band of 40 kDa has been detected which corresponds to the molecular weight of the ~~glycosylated~~ glycosylated clusterin isoform.

Tests of specificity have been performed during primary antibody incubation. Serial dilution of the single peptides have been added during the primary antibody incubation, in order to compete with the bond between the clusterin of the extract and the antibody. The disappearance of the Western Blot signal demonstrates the specificity of the binding, already assessed by the recognition of the protein with a molecular weight of 40 kDa.

Figure 3 shows the specificity of the antibodies for the various clusterin isoforms and demonstrates the diverse isoforms expression

modulation during colon tumourigenesis. In tumoural tissues (T) the isoform that controls the proliferation and is localized in the nucleus of healthy mucosa cells (S) disappears. On the other hand, the cytoplasmic isoform implicated in membrane remodelling and cellular motility increases in tumour.

This assay would give information for the prognosis of disease. In fact, the increase of cytoplasmic clusterin in patients is correlated with colon cancer aggressiveness and correlates with recurrence of metastases in the follow-up.

This investigation could give information on potential formation of recurrences or metastasis in patients.

Example 2: ELISA method for qualitative and quantitative determination of clusterin in biological fluids

The quantitative determination of clusterin/ApoJ in biological fluids (blood, seminal fluid, urine, stool, pleural and ascitic effusions, liquor) according to this invention has been developed by adapting ELISA and RIA techniques for small tumours.

The ELISA method involves the use of two antibodies against ~~glycosilated~~ glycosylated clusterin which is secreted in biological fluids and can be used for the absolute quantitative determination of even minimal variations of clusterin in biological fluids.

A significant improvement has been obtained with the homogeneous immunological dosages that do not need physical separation of the bound fraction of the antigen from the one not bound and so facilitate the automation and speed-up the screening.

The concentration of clusterin is $100 \pm 42 \mu\text{g/ml}$, in healthy individuals.

The method presented in this invention involves a further extension to a RIA dosage that would permit the detection of clusterin in biological fluids, according to the tumour size and its aggressiveness; in fact the RIA dosage is highly sensitive and is therefore indicated to evaluate the differences between the normal levels of clusterin and those slightly increased, due to small tumours or non aggressive ones.

MATERIALS AND METHODS

Standard curve

In order to obtain a standard curve, each peptide used for the generation of each antibody was linked to BSA molecules and immuno-absorbed on 96-well plates in scalar dilutions.

Each peptidic sequence synthesized with the solid phase technique and used for the generation of the oligoclonal antibody against secreted clusterin was then used for the standard curve.

The reference curve was made linking the unmarked peptide to a carrier molecule of known linking capability. Cationic albumin (all the COOH groups are changed in NH₂, PIERCE) was then used. The conjugation of the peptide to the carrier is made with carbodiimide or glutaraldehyde (a NH₂ of the protein is linked to a peptide molecule).

The determination of the not bound NH₂ groups, before and after the peptide conjugation, give the quantity of peptide molecules conjugated per carrier mole. The NH₂ groups are determined with a colorimetric assay.

The standard curve was obtained with increasing quantities of the carrier-peptide compound, from 0 to saturation of each antibody that was maintained constant on plate.

Reactions that have the same quantity of antibody in samples containing the protein or the free peptide will develop an intensity that can be reported on the reference curve for quantization.

The standard curve for determination of clusterin concentration in samples was then performed plating 50 µl of solution per well in TBS + 0,02% BSA + 0,5% Tween-20.

ELISA

The anti-clusterin antibody of this invention used to coat the plates was diluted in a coating buffer (0,05 M carbonate buffer, pH 9,6 + 0,1% NaN₃) at a concentration of 0,5µg/ml and incubated in 96-well plates (50 µl/well), for two hours at 37°C (or overnight at 4°C).

After the incubation, the unbound antibody was removed by washing in TBS (Tris Buffered Saline) + 0,5% Tween-20 and 200 µl of 1% BSA in coating buffer were then added in each well and incubated for 30 minutes at 37°C.

5 The plates were washed in TBS + 0,5% Tween-20.

The serum samples from the patients were plated in three different dilutions (50 µl/well). All the experiments were performed in triplicate.

10 The plates were incubated for 4 hours at 37°C (or overnight at 4°C) and then washed in TBS + 0,5% Tween-20.

A second anti-clusterin antibody was diluted 1:200 in TBS + 0,1% BSA + 0,05% Tween-20 + 2 mM MgCl₂; 50 µl of this solution were added in each well and then incubated for 4 hours at 37°C. The unbound antibody was removed by washing in TBS + 0,5% Tween-20.

15 **Reaction with specific antibodies HRP conjugated**

The plates were incubated for 1 hour at room temperature with 100 µl of anti-rabbit goat IgG conjugated with horseradish peroxidase diluted 1:10000 in 1% BSA/TBS.

20 After the incubation, the plates were washed and the antibody visualized by adding 100 µl/well of detection solution which was incubated for 2 hours at room temperature in the dark; the detection solution was prepared mixing 6 µl H₂O₂, 360 µl tetramethybenzidine (3 mg/ml) dissolved in acetone, 5,64 ml 0,1 M citric acid and 4,36 ml 0,2 M Na₂HPO₄.

25 The reaction was stopped by adding 100 µl 5,3% H₂SO₄ and optical density was read at 492 nm.

RESULTS

30 By the use of the ELISA method validated as described previously, the ability of detecting small quantities of glycosilated glycosylated clusterin in biological fluids, increased quantities in tumoural

cells, and extracellularly secreted clusterin in the intestinal lumen, as well as in blood was evaluated.

Results obtained with this assay are shown in Figure 4: a significant increase of the protein in the culture supernatant fraction of the *ex vivo* isolated tumoural cells, compared to cells from healthy mucosa of the same patient is evident. The specificity of the test is given by the comparison between the protein level in normal and neoplastic cells from the same patient. The culture medium with the same exogenous nutrients has been used as standard. The assay specificity is evident by the results.

In particular, figure 4 shows the clusterin concentration ($\mu\text{g/ml}$) evaluated by ELISA in the culture supernatant of the *ex vivo* isolated cells from healthy and neoplastic colonic mucosa. The complete culture medium was used as control. The normal (10×10^7 cells) and neoplastic (10×10^7 cells) cells from colonic mucosa were incubated two days at 37°C in complete medium. The culture supernatant fraction was dosed by the ELISA assay.

The aim of the diagnostic kit according to this invention is to determine the clusterin levels in biological fluids in an absolute quantitative, specific and selective way, thus permitting a timing and not invasive diagnosis of tumours.

Specifically for colorectal cancer, the increase of glycosylated clusterin expression in the cytoplasm correlates with the tumour aggressiveness and metastatic potential. Therefore this kit can be also used for the prediction of stage and grade of the malignant disease.

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SUMMARY

The invention concerns anti-clusterin oligoclonal antibodies able to recognize and bind in a selective and specific way antigenic epitopes of clusterin isoforms to be used in tumours diagnosis and in the prediction of their malignancy grade, diagnostic method and related kits.

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